

INTERACTION OF CHLORSULFURON TREATMENT AND IRON DEFICIENCY OR EXCESS IN YOUNG PEA PLANTS

Nenova V. , K. Kocheva*

Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Bldg. 21, 1113 Sofia, Bulgaria

Received: 17 October 2014 Accepted: 05 December 2014

Summary: Nutrient stress may modify plant reaction to herbicide treatment. On the other hand, herbicides might induce or aggravate nutrient disorders. The separate and combined effects of chlorsulfuron (CIS) and iron supply on growth, photosynthetic pigment content and functional state of the photosynthetic apparatus, assessed by chlorophyll fluorescence measurements, were compared. Young pea plants were grown hydroponically in a growth chamber, supplied with iron, ranging from complete deficiency to excess (0, 2- optimum concentration, 10 or 50 mg.l⁻¹ Fe) and sprayed with CIS (0. 10⁻⁶ or 10⁻⁵M). Both Fe deficiency and strong Fe excess reinforced the inhibitory effect of CIS on shoot and root biomass, while low Fe excess (10 mg.l⁻¹ Fe) slightly mitigated the herbicide effect on growth, but affected negatively PSII activity. Separately, Fe deficiency and CIS decreased the chlorophyll and carotenoids content. Due to a concentration effect, when both factors were in combination the chlorosis of Fe deficient plants as well as the imbalance between the pigments were less pronounced. Fe deficiency was the individual stress with the strongest negative effect on the photosynthetic apparatus when estimated by chlorophyll fluorescence. In combination with CIS, this effect was softened. The negative effect of CIS on the fluorescence parameters was evident at 10⁻⁵M and was reinforced when combined with 50 mg.l⁻¹ Fe. Individual stressors as well as their combinations caused an increase in electrolyte leakage from leaf tissue, which was an indicator for the occurrence of cell membrane injury. It was concluded that the effect of CIS depended not only on its concentration, but also on Fe supply.

Citation: Nenova V., K. Kocheva, 2014. Interaction of chlorsulfuron treatment and iron deficiency or excess in young pea plants. *Genetics and Plant Physiology*, Conference “Plant Physiology and Genetics – Achievements and Challenges”, 24-26 September 2014, Sofia, Bulgaria, Special Issue (Part 2), 4(3–4): 140–154.

Keywords: Chlorsulfuron; combined stress; Fe deficiency; Fe excess; herbicides; *Pisum sativum* L.

Abbreviations: ALS – acetolactate synthase; Chl. – chlorophyll; CIS – chlorsulfuron; DM – dry matter; DMC – dry matter content; Fe – iron; IGA – index of growth alteration; PSII – photosystem II.

*Corresponding author: nenova@bio21.bas.bg

INTRODUCTION

Herbicides are widely used in agriculture for weed control, but they might decrease yield because of injuries on cultivated plants. The proper selection of herbicide type, rates, mode and time of application is crucial for the final outcome. Additional factors might modify the effect, nutrient supply being one of them. Recently, special attention has been drawn on the most widely used herbicide, glyphosate, including its impact on plant nutrition (Cakmak et al., 2009). Otherwise, the herbicide-nutrient interactions have not received much attention with the exception of the mutual relations of chlorsulfuron (CIS) and Zn or Cu supply in wheat. Chlorsulfuron is a sulfonylurea herbicide belonging to the group of ALS-inhibitors which block the first step in the synthesis of branched-chain amino acids (Zhou et al., 2007). Osborne and Robson (1993) found that luxury nutrient supply was more favorable for plant growth and degree of recovery after CIS application. Increased Zn concentrations could alleviate or prevent the deleterious effect of CIS on root growth (Wheal et al., 1998). On the other hand, CIS might induce or aggravate Zn and Cu deficiency in wheat (Dong et al., 1995; Tang and Robson, 2000). Soil application of the herbicide decreased the concentrations of a range of elements in shoots, especially during early growth, Zn and Cu being most affected among the studied microelements, followed by Mn and subsequently by Fe (Osborne et al., 1993; Osborne and Robson, 1993). It appeared to affect Zn, Cu and Mn accumulation by inhibiting growth of fine roots and specifically influencing micronutrient transport systems (Rengel

and Wheel, 1997). The effect of CIS on growth and nutrient uptake was genotype-dependent and associated with sensitivity to Cu or Zn deficiency (Dong et al., 1995; Tang and Robson, 2000).

Often herbicide application has to be done on plants exposed to iron supply far from the optimum due to adverse soil, climatic or anthropogenic conditions. Soil conditions, causing insufficient or excess Fe uptake, for instance calcareous or acid soils and water logging, are widespread in nature. The information about chlorsulfuron-iron interactions in plants is limited, though some field observations suggest its existence. As the physiological activities of dicots and grasses in response to Fe-deficiency are different (Jolley et al., 2004; Hindt and Guerinot, 2012) one might expect diversity in their reactions as well. According to Franzen et al. (2004) some ALS-inhibitors may have potential for greater injury of soybean under soil and environmental conditions leading to Fe-deficiency chlorosis. Foliar Fe sprays were demonstrated to antagonize their effect, but lower crop injury was accompanied with poorer weed control (Franzen et al., 2003). According to Jolley et al. (2004) research directed to clarify the variation of stress responses during stress interaction might contribute to a broader physiological explanation of some field observations.

The objective of this study was to investigate the interaction of Fe supply and foliar CIS treatment on young pea plants. The individual effects of stressors on growth, photosynthetic pigments content and functional state of PS2 estimated by means of prompt chlorophyll fluorescence were compared, and on this basis variations in their combined effects

were outlined with respect to possible Fe impact on herbicide effect.

MATERIALS AND METHODS

Pea plants (*Pisum sativum* L. cv. Manuela) were grown hydroponically in a growth chamber on half-strength Hoagland-Arnon solution I with micronutrient supply according to a modified Hoagland's "A-Z" solution (Nenova, 2009). Iron was supplied in the form of ethylenediaminetetraacetic acid iron (III)- sodium salt hydrate (Fluka) at 4 concentrations: 0 mg.l⁻¹ (complete Fe deficiency), 2 mg.l⁻¹ - optimum, 10 mg.l⁻¹ (slight excess) and 50 mg.l⁻¹ (strong excess). On day 10 after seed soaking cotyledons were removed in order to reduce nutrient supply from the seed. On day 22 (after the appearance of first visual chlorosis signs in Fe-deficient plants) plants were sprayed with water solutions of herbicide Glean®FC (DuPont Crop Protection) containing 75% chlorsulfuron as the active ingredient, with addition of 0.05% Tween 80 as a surfactant. During the foliar application CIS was prevented from entering the nutrient solution. Three concentrations CIS (0, 10⁻⁶ M or 10⁻⁵ M) were applied. Plants grown at 2 mg.l⁻¹ Fe in the absence of CIS were designated as control.

On day 37 fresh biomass of shoots and roots as well as the length of shoots and primary root were measured. The dry matter content (DMC) in g DM per 100 g fresh biomass was determined after drying the plant material at 105 °C to a constant weight and the values were used to calculate the dry matter per plant (DM). The shoot/root ratio was calculated by using the respective values for DM. Using

shoot and root length, DM and fresh biomass, an Index of growth alteration (IGA) was calculated as

$$IGA = \frac{1}{n} \sum_{i=1}^n \frac{(P_{si} - P_{ci})}{P_{ci}} \times 100$$

where P_{si} was the established value of the respective *i*th parameter of the stressed plants, P_{ci} was the established value of the respective *i*th parameter of the control plants, n=6 (number of the used morphological parameters).

The youngest fully expanded leaves were used for further analyses. The concentrations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids were measured after extraction in 80% acetone and values were calculated according to McKinney (1941). Prompt chlorophyll fluorescence was measured on a pulse modulation chlorophyll fluorometer (PAM 101; H. Walz' Effeltrich' Germany) using saturating light at 3500 μmol m⁻²s⁻¹ and actinic light at 330 μmol m⁻²s⁻¹ PPFD. The minimum chlorophyll fluorescence yield in the dark-adapted state and in the light-adapted state (F₀ and F₀' , respectively), maximum chlorophyll fluorescence yield in the dark-adapted state and in the light-adapted state (F_m and F_m' , respectively), and steady-state chlorophyll fluorescence (F_s) were recorded. The following parameters were calculated according to Roháček (2002): maximum variable chlorophyll fluorescence yield in the dark-adapted state (F_v = F_m - F₀); potential maximum quantum yield of PSII (F_v/F_m); actual quantum yield of PSII [Φ_{PSII} = (F_m' - F_s)/F_m']; coefficient of photochemical quenching of the variable chlorophyll fluorescence based on a 'puddle' model [q_p = (F_m' - F_s)/(F_m' - F₀')]; effective quantum yield of PSII photochemistry [Φ_{exc} = (F_m' -

F_0'/F_m']; non-photochemical chlorophyll fluorescence quenching [$NPQ = (F_m - F_m')/F_m'$]. The coefficient of photochemical quenching (q_L) that measures the fraction of open PSII centers based on a 'lake' model for PSII, the quantum yield for dissipation by down regulation in PSII (Φ_{NPQ}), and the quantum yield of non-regulated energy dissipated in PSII (Φ_{NO}) were calculated according to Kramer et al. (2004):

$$q_L = q_p(F_0'/F_s);$$

$$\Phi_{NPQ} = 1 - \Phi_{PSII} - \frac{1}{NPQ + 1 + q_L(F_m'/F_0' - 1)};$$

$$\Phi_{NO} = \frac{1}{NPQ + 1 + q_L(F_m'/F_0' - 1)}.$$

For ion leakage kinetics, leaf discs from each treatment were soaked in distilled water at room temperature. Conductivity of the solutions was measured at multiple time points during a 24-h incubation period and again after boiling the samples for 30 min. Results were expressed as the ratio κ/κ_{\max} versus time, where κ is conductivity of samples at a particular moment and κ_{\max} is total electrolyte content determined after boiling. Hence, a multiple-point kinetics curve was obtained. Fitting of experimental data was performed by the Exponential Associate function of Origin 5.0 software:

$$\kappa/\kappa_{\max} = A_1(1 - e^{-t/t_1}) + A_2(1 - e^{-t/t_2}) \quad (\text{Kocheva et al., 2014}).$$

Six independent experiments were conducted with different combinations of the experimental treatments, each in at least 3 replicates. Each biological replicate was conducted in 3-4 technical replicates. An average of all experiments

was presented and Statgraphics Plus (version 2.1) was used in order to discriminate among the means by Fischer's least significant difference procedure. Values followed by the same letter are not significantly different at the 95% confidence level.

RESULTS

Growth

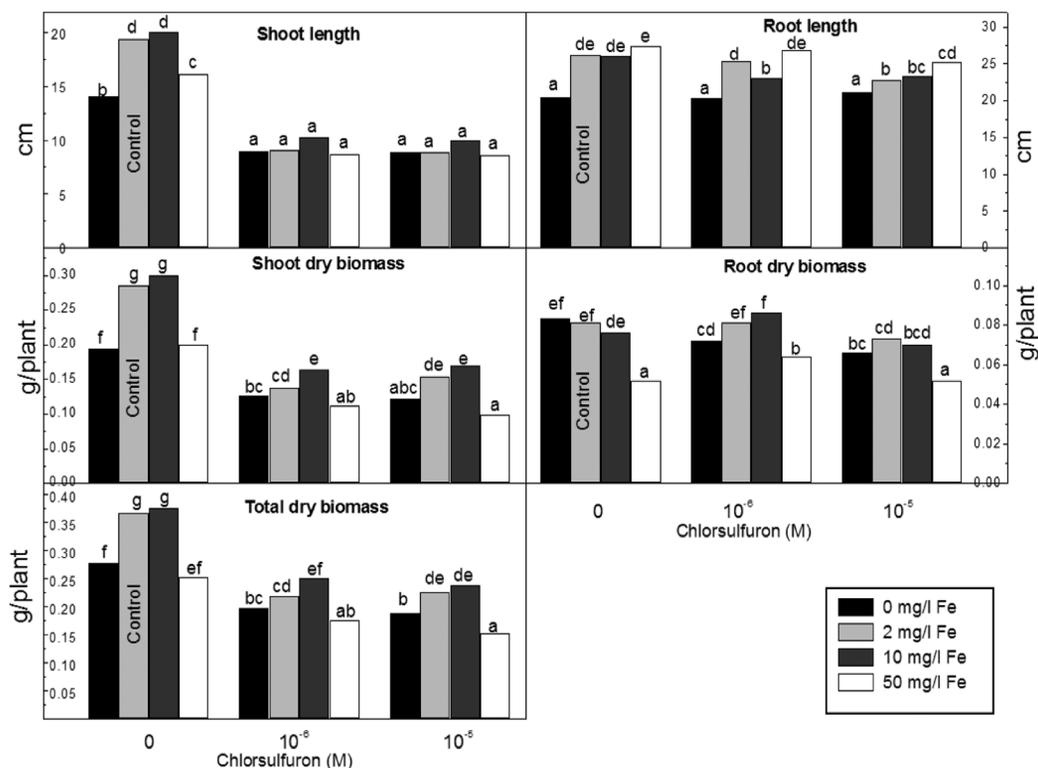
Inadequate Fe supply and CIS treatment when applied separately, affected plant growth and development. At the end of the experiment control plants had 11 compound leaves, those supplied with 0 or 50 mg.l⁻¹ Fe ended up with 10 leaves at average, while CIS spray stopped leaf morphogenesis at 6 leaf stage regardless of Fe concentration. Low Fe excess did not change growth, while complete Fe deficiency and 50 mg.l⁻¹ Fe decreased IGA by 22-24% (Table 1). They both had a similar effect on shoot DM (decreased by around 30%) and shoot length (decreased by around 20-30%, the effect of deficiency being stronger) (Fig. 1). As the DMC in shoots did not vary significantly (Table 1), the changes in shoot DM reflected those in fresh biomass (data not shown). These stressors differed in their effect on roots. Iron deficiency shortened the roots, but did not alter the root DM (though the decreased by 24% fresh biomass) because of increased root DMC. Thus, a significant decrease of the shoot/root ratio in Fe deficient plants was found. Strong Fe excess did not alter root length, raised only slightly DMC, decreased root biomass by around 40%, and affected insignificantly the shoot/root ratio. Individual CIS treatment had a stronger

Table 1. Effect of Fe supply and chlorsulfuron (CIS) on Index of growth alteration, shoot/root ratio and dry matter content (DMC) in shoots and roots of pea plants.

	0 M CIS			10 ⁻⁶ M CIS			10 ⁻⁵ M CIS		
	Index of growth alteration (%)			Shoot / root ratio					
0 mg.l ⁻¹ Fe	-22.0±3.0	-42.0±2.0	-44.0±2.0	2.3±0.3	1.7±0.3	1.8±0.3			
2 mg.l ⁻¹ Fe		-33.0±3.0	-37.0±2.0	3.5±0.4	1.7±0.2	2.1±0.2			
10 mg.l ⁻¹ Fe	0.0±3.0	-29.0±2.0	-35.0±2.0	3.9±0.6	1.9±0.1	2.4±0.2			
50 mg.l ⁻¹ Fe	-24.0±3.0	-41.0±2.0	-48.0±2.0	3.8±0.3	1.7±0.1	1.9±0.1			
	Shoot dry matter content (%)			Root dry matter content (%)					
0 mg.l ⁻¹ Fe	9.4±0.4	13.1±0.5	16.0±2.0	6.9±0.3	7.5±0.5	7.3±0.7			
2 mg.l ⁻¹ Fe	9.7±0.5	12.9±0.7	14.8±1.2	5.1±0.3	6.7±0.4	6.9±0.2			
10 mg.l ⁻¹ Fe	9.7±1.0	13.3±0.8	15.4±0.6	5.2±0.2	6.9±0.4	6.7±0.3			
50 mg.l ⁻¹ Fe	9.5±0.3	12.7±0.4	13.4±1.0	5.5±0.3	7.0±0.4	7.2±0.3			

effect on growth than non-optimal Fe supply, IGA being -33% and -37% under 10⁻⁶ M and 10⁻⁵ M, respectively. A further increase of the concentration to 10⁻⁴ M led

to lower IGA of -42% (data not shown). This greater inhibition of growth was attributed mainly to the greater inhibition of shoot growth, which resulted in

**Figure 1.** Effect of iron supply and chlorsulfuron on shoot and root length, shoot and root dry biomass, and total dry biomass of pea plants. Values with the same letter are not significantly different at $p=0.05$ according to Fischer's LSD test.

decreased shoot/root ratios. The length and the DM of shoots at 10^{-6} and 10^{-5} M CIS decreased by 45-55%, with insignificant differences between both concentrations. The decrease in fresh biomass was even greater (by 70%), because of the higher shoot DMC. The latter increased more at 10^{-5} M than at 10^{-6} M. The herbicide effect on roots was less pronounced, but the differences between both concentrations were more evident. The root length and DM decreased only at the higher concentration applied.

When combined with Fe deficiency CIS decreased IGA from -33 to -42% at 10^{-6} M, and from -37 to -44% at 10^{-5} M. The additional negative effect of Fe deficiency on shoot DM and DMC was manifested better at the higher concentration. The typical for Fe deficiency shortening of roots was evident also under both CIS treatments. Because of the additional rise of root DMC the decrease in root DM was significant only at 10^{-6} M, though root fresh biomass decreased at both concentrations. Fe deficiency caused an additional decrease by 17% of total DM of plants sprayed with 10^{-5} CIS. The decrease observed at the low CIS concentration was by 10% at average and not always significant. The highest IGA decrease (-48%) was observed when 10^{-5} CIS was combined with 50 mg.l^{-1} Fe. Both shoot and root biomass were decreased, resulting in a drop of total DM by around 20% under 10^{-6} M CIS, and by 32% - under 10^{-5} M CIS, as compared to the respective individual CIS treatments. In general, the combination of CIS application with Fe deficiency or strong excess did not modify significantly the shoot/root ratio, as compared to the separate herbicide treatment. When the lower CIS treatment

was combined with 10 mg.l^{-1} IGA, shoot DM and total DM were less decreased than at 10^{-6} M CIS alone, e.g. the total DM was higher by 14%. A slight similar trend was observed also at 10^{-5} M CIS. The shoot/root ratio was also higher, though far below the control value.

Photosynthetic pigments

The first visual chlorosis signs in the Fe deficient plants (more light green color of young leaves) appeared between days 19-22. At the end of the experiments the chlorophyll and carotenoid contents in the youngest fully developed leaves of these plants were decreased by around 80 and 65%, respectively, thus resulting in a decreased chlorophylls/carotenoids ratio (Fig. 2). Besides, the Chl *a*/Chl *b* ratio rose. No significant changes in pigments content and balance were found under Fe excess. CIS treatment also decreased the pigments content by 30-35%, but did not alter their ratios. A significant decrease of the Chl *a*/Chl *b* ratio by 11% was observed only at 10^{-4} M CIS, though no further decrease of total chlorophyll content was observed (data not shown).

The combination of CIS with Fe deficiency caused a further decrease of photosynthetic pigments as compared to the individual CIS treatment. Though, in comparison to plants suffering from Fe deficiency alone, the chlorophyll content increased more than twice, the carotenoids increased by only 25-45%, and the balance between the pigments was restored. The combination of CIS with excess Fe affected insignificantly the pigment content with the exception of the combination of the highest CIS and Fe concentrations, which raised the carotenoid content above that of the single CIS application.

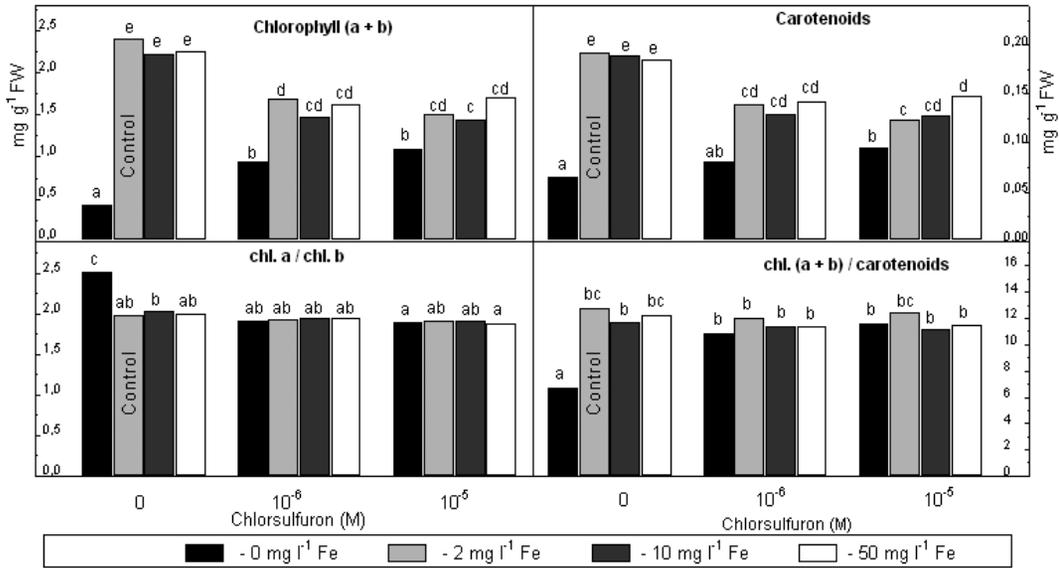


Figure 2. Effect of iron supply and chlorsulfuron on chlorophyll (*a* + *b*) and carotenoids content, and on chlorophyll *a*/chlorophyll *b* and chlorophyll (*a* + *b*)/carotenoids ratios in the youngest fully expanded leaves of pea plants. Values with the same letter are not significantly different at $p=0.05$ according to Fischer's LSD test.

Chlorophyll fluorescence

When applied separately Fe deficiency and 10^{-5} M CIS were the stressors with the greatest impact on chlorophyll fluorescence parameters (Fig. 3). In Fe deficient plants the actual quantum yield of PSII (Φ_{PSII}) decreased by 16%, mainly due to the lower fraction of open PSII reaction centers, as estimated by lower photochemical quenching- q_p and q_L which decreased by 14% and 18%, respectively. The portion of non-regulated heat dissipation (Φ_{NO}) increased by almost 80%, while the regulated heat dissipation (estimated by Φ_{NPQ} and NPQ) decreased. Both ratios F_v/F_m and F_v/F_0 characterizing the dark-adapted state diminished by 12% and 45%, respectively. At a concentration of 10^{-5} M CIS Φ_{PSII} decreased by 20%, but mainly due to a 14% decrease of the effective quantum yield of PSII (Φ_{exc}). Besides, q_p also tended to be lower. Both

parameters estimating the regulated heat dissipation increased by 20%, while Φ_{NO} did not change significantly. F_v/F_0 dropped by 14%, while F_v/F_m was not altered. The lower CIS concentration tended to have similar, but less pronounced effect with no significant differences from control values. The strong Fe excess decreased both F_v/F_0 and NPQ, whereas Φ_{NO} was slightly increased.

Fe deficiency did not modify significantly the effect of CIS on the fluorescence parameters with two exceptions, concerning the low herbicide concentration: an increase in Φ_{NO} , and a decrease in F_v/F_0 , which were not altered by the individual 10^{-6} M treatment. When compared to the individual Fe deficiency treatment F_v/F_m , F_v/F_0 , Φ_{NPQ} and NPQ increased, while Φ_{NO} was found to decrease. Under the low CIS treatment slight Fe excess also tended to decrease both ratios characterizing the dark-adapted

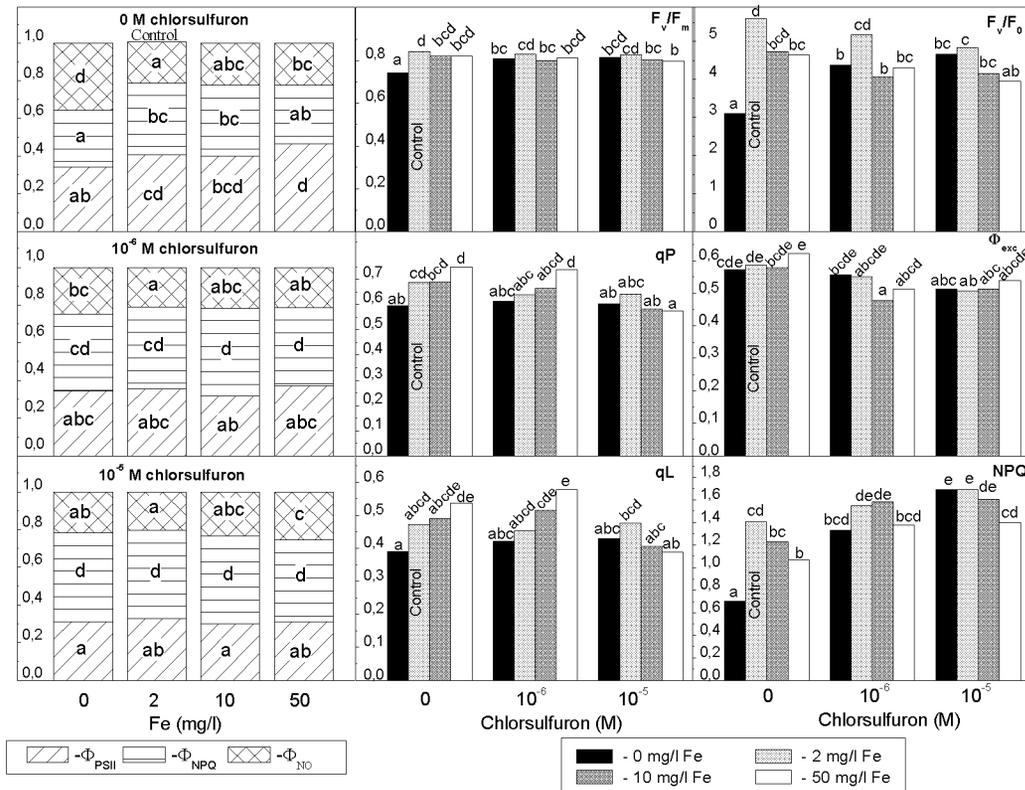


Figure 3. Effect of iron supply and chlorsulfuron on chlorophyll fluorescence parameters in the youngest fully expanded leaves of pea plants. Left panel: Yields for dissipative processes for the energy absorbed by PSII, where Φ_{PSII} - yield of the photochemistry, Φ_{NPQ} - yield for dissipation by down regulation, Φ_{NO} - yield of non-regulated energy dissipation. q_p and q_L - coefficients of photochemical quenching of the variable chlorophyll fluorescence, based on a 'puddle' or on a 'lake' model, respectively. F_v/F_m - maximum quantum yield of PSII; F_v/F_0 - indicator of maximum efficiency of photochemical processes in PSII and/or the potential photosynthetic activity; Φ_{exc} - effective quantum yield of PSII; NPQ - non-photochemical chlorophyll fluorescence quenching. Values with the same letter are not significantly different at $p=0.05$ according to Fischer's LSD test.

state. The strong Fe excess had a similar effect at 10^{-5} M CIS, and in addition it also increased Φ_{NO} by 25%.

Electrolyte leakage

Electrolyte leakage kinetics from leaf tissue was used to estimate cell membrane stability in some of the treatments (Kocheva et al., 2014). Though differences were not big, four groups of curves might be discerned (Fig. 4). The lowest electrolyte leakage was registered

in control plants and in Fe-deficient plants sprayed with 10^{-5} M CIS. Higher leakage was detected in plants supplied with optimum Fe and receiving the lower CIS treatment. Next followed a group of four curves which reflected the effect of three of the individual stressors: Fe deficiency, strong Fe excess and 10^{-5} M CIS, as well as the combination of 10^{-6} M CIS and 50 mg.l^{-1} Fe. The greatest leakage was observed under the combination of 10^{-5} M CIS and 50 mg.l^{-1} Fe.

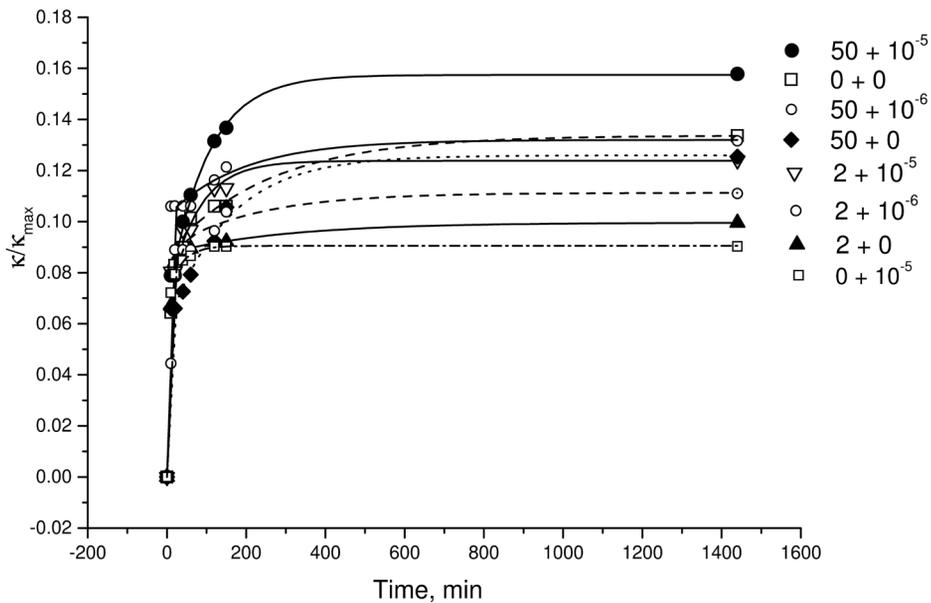


Figure 4. Effect of iron supply and chlorsulfuron on electrolyte leakage kinetics in the youngest fully expanded leaves of pea plants.

DISCUSSION

Individual effect of stress factors

Chlorsulfuron treatment, Fe deficiency and Fe excess, when applied separately altered in a different manner the physiology, development and growth of pea plants. In our detailed earlier studies, we demonstrated that when plants were not supplied with an optimum amount of Fe, growth inhibition and physiological changes developed gradually, depending on the strength and duration of the imposed stress with some peculiarities depending on plant genotype (Nenova, 2006; 2009). The present study confirmed our earlier results. The observed loss and imbalance of photosynthetic pigments under Fe deficiency might be attributed not only to hampered chlorophyll biosynthesis, which has several steps under Fe control, but also to the loss of other thylakoid constituents (Terry and

Abadia, 1986). The preferential loss of Chl *b* was indicative of a smaller light-harvesting complex in the photosynthetic unit, while the decreased carotenoids/chlorophylls ratio most likely resulted from smaller alteration in the content of lutein and xanthophylls (Larbi et al. 2006). These changes might partially increase photoprotection of highly chlorotic leaves by decreasing excessive light absorption and enhancing its controlled dissipation, as well as by ensuring some antioxidant protection. Nevertheless, instead of a rise of regulated thermal dissipation, we observed a decrease, probably due to low light conditions in our growth chamber (Kramer et al., 2004). At the same time, the actual quantum yield of PSII predicting the linear electron transfer also decreased, thus being at least partially the cause for a lower photosynthetic rate (Larbi et al. 2006; Nenova, 2009). The rise of non-regulated

heat dissipation compensated for the drop of the above-mentioned two dissipative fluxes. The observed reduction of both ratios characterizing the dark-adapted state, usually regarded as a measure of photoinhibition, according to Morales et al. (2001) might be overestimated in the case of Fe-deficiency.

In addition to low photosynthesis, growth might be affected also by Fe deficiency directly or indirectly through other mechanisms, i.e. by alteration of nucleic acid metabolism, respiration, nutrient and phytohormonal balance, etc. Biomass allocation towards the roots as well as growth depression is not specific for Fe deficiency and can be observed under different nutritional disorders. It could be associated with differences in the initiation of primordia and activity of meristems, with changes in structure and storage in cells, and with differences in the partitioning and incorporation of photoassimilates into shoot and root tissues (McDonald et al., 1996). In the case of Fe deficiency, plants respond by morphological changes such as enhanced lateral root formation, increased formation and branching of root hairs, root-tip swelling which as a whole result in an increased root surface area for the reduction and uptake of Fe (Hindt and Gueriot, 2012).

Under strong Fe excess, the extent of growth depression was comparable to that caused by Fe deficiency. It was not associated with significant biomass allocation. Oxidative stress caused by increased reactive oxygen species (ROS) production is regarded as the main mechanism of Fe toxicity. In plants the balance between Fe availability for metabolism and its sequestration to

avoid subsequent ROS-related damages is established by modulation of the expression of the iron storage protein ferritin (Briat et al., 2010). Compared to other studies, growth depression observed under treatment with 50 mg.l⁻¹ Fe might not be attributed to direct Fe toxicity but rather to impeded uptake of other nutrients (Batty and Younger, 2003). In comparison to our previous studies, stress was not prolonged enough to cause alterations in pigment content under strong Fe excess, and growth depression under 10 mg.l⁻¹ Fe. The latter concentration was chosen as slightly toxic as it hampered the growth on day 41 of cv. Manuela, and on day 34 of cv. Ran-1 (Nenova, 2006; 2009).

These two stress factors increased to a similar extent the electrolyte leakage from leaves, indicating that the integrity and stability of cell membranes and cell walls were affected. As earlier we demonstrated that Fe deficiency and excess had opposite effects on specific leaf area, the former resulting in thinner leaves, while the latter - in small thick leaves (Nenova, 2006), one might speculate that alterations in leaf morphology might not be determinant for the observed effect. On the other hand, both Fe deficiency and excess are supposed to induce oxidative stress and lipid peroxidation (Briat et al., 2010; Jelali et al., 2012). These stressors had a slight effect on plant development by diminishing the number of leaves by one.

This was not the case with the individual CIS treatment, which reduced the leaf number by five, together with a much stronger decrease in growth, especially of shoots where the herbicide was applied. Shoots withering (as pointed by the great increase of DMC) contributed

partially to the observed decrease of shoot DM, but the main reason had to be sought in the activity of CIS as a strong and rapid inhibitor of cell division. According to Ray (1982) the reduction of growth after CIS application was closely associated with cell division inhibition and occurred at concentrations where photosynthesis, respiration, RNA synthesis and protein synthesis were unaffected. Though ALS enzyme is without doubt the site of action of ALS-inhibiting herbicides, their effect could not be mainly explained by valine, leucine and isoleucine starvation (Zhou et al., 2007). Clayton et al. (1991) supposed that in root tip cultures of pea the CIS effect on cell cycle activity might be explained by accumulation of intermediates of the branched-amino-acid pathway. In our experiments, CIS was prevented to enter the nutrient solution. Though root entry of the herbicide leads to greater crop injury than foliar entry, its uptake by foliage may still reduce root growth, and thus impair the ability of plants to take up nutrients (Lemerle and Cousens, 1993). Structural changes of nuclei and mitochondria in the root meristematic cells of pea plants were observed quickly after CIS treatment suggesting a decrease of transcriptional and translational activity (Stoynova et al., 1997). Root growth inhibition was not caused by sugar starvation as in addition to carbohydrate accumulation in leaves, accumulation of sucrose and/or starch in roots was also detected (Zabalza et al., 2004). In the present study, growth changes induced by CIS occurred in a dose-dependent manner. So did changes in electrolyte leakage. Kapchina-Toteva et al. (2004) found that in tobacco leaves the cell membrane damage, assessed by malondialdehyde accumulation, depended

on plant tolerance to CIS and in the sensitive line it increased gradually in response to herbicide treatment. Though decreased as compared to the control, the chlorophyll content in our experiments was higher in comparison to Fe deficient plants. Leaf yellowing and decreased photosynthetic rates are regarded as secondary effects of CIS treatment which follow the growth arrest (Sousa et al., 2014). Usually chlorosis starts in the newest leaves as this is where the greatest synthesis of new amino acids is needed (Hanson, 2014). On the other hand, structural disturbances of pea chloroplasts similar to those observed during senescence were observed (Stoynova et al., 1997) thus suggesting that chlorophyll breakdown during chloroplast disassembly took place. A decrease of the Chl *a*/Chl *b* ratio, suggesting an increase in the size of the antenna was observed only at 10^{-4} M CIS. A similar increase was observed by Sousa et al. (2014) when the herbicide treatment resulted in almost inactive reaction centers and large heat dissipation. The authors supposed that antenna size was increased in an attempt to compensate for the great dissipation of excitation energy. In opposite to chlorotic Fe deficient plants, we also observed high regulated energy dissipation caused by 10^{-5} and 10^{-4} M CIS which was combined with a decline in photochemical efficiency, but the changes were not so severe in order to accept the explanation of Sousa et al. (2014).

Combined effect of stress factors

Chlorsulfuron increased the pigment content and restored the pigment balance in the Fe-deficient plants as compared to the chlorotic plants which were not sprayed. While the slight increase of carotenoids

might be explained by a concentration effect, i.e. similar carotenoid amount for smaller leaf biomass, the greater increase of chlorophylls should be due to its less hampered synthesis. Most likely, the reduction in leaf growth resulted in a higher physiologically active Fe concentration when expressed on a dry matter basis. This assumption was further supported by the mitigation of the negative effect of Fe deficiency on fluorescence parameters caused by CIS. Nevertheless, under the combined stress the plants continued to suffer from deficiency of active Fe, which indirectly was confirmed by an additional pigment decrease as compared to the separate CIS treatments. Iron deficiency potentiated the effect of CIS as a growth inhibitor. The effect on shoots was more pronounced at the high CIS concentration, and on roots - at the low concentration, which, on its own did not alter roots. Fe-deficiency (and slight excess, as it will be demonstrated later) increased the susceptibility of PSII to 10^{-6} M CIS, so that the response was similar to the response to 10^{-5} M CIS. Pea plants respond to Fe deficiency by significant metabolic rearrangements aiming to ensure not only the already mentioned morphological changes in root system, but also - increased solubility, reduction and absorption of iron (Jelali et al., 2010). One might speculate that the stress-coping mechanisms and the capacity of damage repair had been exhausted thus rendering the plant susceptible to the additional stress which, in this case was the herbicide treatment (Alexieva et al., 2003).

Another possible mechanism of interaction between these stress factors is through shikimate and phenylpropanoids

synthesis pathways. Higher concentrations of phenolics and flavonoids were observed in leaf and root tissues of Fe-deficient pea plants, and the activities of shikimate pathway enzymes were enhanced. The increased level of phenolics contributed to Fe mobilization in roots, and was related to plant tolerance to Fe deficiency (Jelali et al., 2012). On the other hand, Orcary et al. (2011) observed induction of the first enzyme of the shikimate pathway by CIS, as well, and suggested an important role for hydroxycinnamic acids accumulation in the mode of action of ALS-inhibitors. Though phenolics have a role in antioxidant protection and lignification of the cell wall, as already mentioned both Fe deficiency and CIS are known to induce oxidative stress and membrane damages. So we did not expect the electrolyte leakage under their combination to be the same as in control plants.

On the other hand, glyphosphate, a herbicide which alters protein metabolism by breaking the biosynthesis of aromatic amino acids, was shown to inhibit Fe root uptake especially under Fe deficient conditions, to hamper root to-shoot transport of Fe, as well as phloem loading and phloem transport of Fe into sink organs (Ozturk et al., 2008; Cakmak et al., 2009). These effects were attributed to the Fe-complexing ability of glyphosate. We considered the possibility for a similar interaction between Fe and CIS, based on the recommendations not to apply CIS together with most trace elements because of the formation of stable metal salts, but having in mind that in nutrient solution and in plants Fe was in a chelated form. Wheal and Rengel (1997) demonstrated that CIS did not decrease Zn uptake from chelate-buffered

solutions when applied at the moment of absorption, and a pretreatment of at least 3 days was required for CIS applied to the roots to decrease Zn uptake.

We tested the possibility to counteract the eventual CIS-induced Fe-deficiency by increasing the external Fe supply. Indeed, we observed a slight positive effect of mild Fe-excess on growth at the low CIS concentration. This effect, however, was accompanied with a decrease of potential maximum quantum yield of PSII, which was not affected by both individual stress factors. This implies that probably photoinhibition, i.e. irreversible photoinactivation of PSII which could be overcome only after *de novo* protein synthesis, took place.

Similarly, strong Fe excess decreased the F_v/F_m ratio in plants sprayed with 10^{-5} M CIS, and in addition increased the non-regulated heat dissipation, usually regarded as a loss process due to PSII inactivity. An additional growth inhibition took place at both CIS concentrations. An increased production of ROS which are detrimental to all biological structures is a common plant response to different stress factors, including metal toxicity and herbicide treatment. If regarding the extent of membrane damages, estimated by electrolyte leakage as an indirect marker for oxidative stress, the observed additional rise of electrolyte leakage supported the idea that plants subjected to the combined action of 50 mg.l⁻¹ Fe and 10^{-5} M CIS experienced the strongest stress, which also resulted in the greatest growth inhibition. Cross-synergism between the factors occurred, as their cumulative effect exceeded the simple additive effect of their individual action (Alexieva et al., 2003).

REFERENCES

- Alexieva V, S Ivanov, I Sergiev, E Karanov, 2003. Interaction between stresses. *Bulg J Plant Physiol*, XXIX: 1–17.
- Batty L, P Younger, 2003. Effects of external iron concentration upon seedling growth and uptake of Fe and phosphate by the common reed, *Phragmites australis* (Cav.) Trin ex. Steudel. *Annals of Botany*, 92: 801–806.
- Briat J-F, K Ravet, N Arnaud, C Duc, J Boucherez, B Touraine, F Cellier, F Gaymard, 2010. New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants. *Annals of Botany*, 105: 811–822.
- Cakmak I, AYazici, Y Tutus, L Ozturk, 2009. Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. *Europ J Agronomy*, 31: 114–119.
- Clayton D, T Reynolds, 1991. Chlorsulfuron effects on protein synthesis and accumulation in cultured root tips of *Pisum sativum* L. *J Plant Physiol*, 137: 337–341.
- Dong B, Z Rengel, R D Graham, 1995. Effects of herbicide chlorsulfuron on growth and nutrient-uptake parameters of wheat genotypes differing in Zn-efficiency. *Plant Soil*, 173: 275–282.
- Franzen D W, J H O'Barr, R K Zollinger, 2004. Influence of certain postemergence broadleaf herbicides stressed from iron deficiency chlorosis. *Agronomy J*, 96: 1357–

- 1363.
- Franzen D W, J H O'Barr, R K Zollinger, 2003. Interaction of foliar application of iron HEDTA and three postemergence broadleaf herbicides with soybean stressed from chlorosis. *J Plant Nutr*, 26: 2365–2374.
- Hanson B, 2014. Herbicide symptomology photos from weed science workshop. UC Weed Science, Weed control, management, ecology, and minutia. Published on line on July 14, 2014. Accessed on October 10, 2014 from <http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=14633>.
- Hindt M, M L Guerinot, 2012. Getting a sense for signals: Regulation of the plant iron deficiency response. *BBA - Molecular Cell Research*, 1823: 1521–1530.
- Jelali N, S Moez, W Dhifi, W Mnif, C Abdelly, M Gharsalli, 2012. Secondary metabolism responses in two *Pisum sativum* L. cultivars cultivated under Fe deficiency conditions. *African Jof Biotechnol*, 11: 14828–14836.
- Jelali N, M Wissal, M Dell'orto, C Abdelly, M Gharsalli, G Zocchi, 2010. Changes of metabolic responses to direct and induced Fe deficiency of two *Pisum sativum* cultivars. *Env Exp Bot*, 68: 238–246.
- Jolley V D, N C Hansen, A K Shiffer, 2004. Nutritional and management related interactions with iron-deficiency stress response mechanisms. *Soil Sci Plant Nutr*, 50: 973–981.
- Kapchina-Toteva V, S Slavov, R Batchvarova, A Krantev, D Stefanov, A Uzunova, 2004. Stress markers in chlorsulfuron tolerant transgenic tobacco plants. *Bulg J Plant Phys*, 30: 103–111.
- Kocheva K, G Georgiev, V Kochev, 2014. An improvement of the diffusion model for assessment of drought stress response in plants. *Physiol Plant*, 150: 88–94.
- Kramer D M, G Johnson, O Kiirats, G E Edwards, 2004. New fluorescence parameters for the determination of Q_A redox state and excitation energy fluxes. *Photosynth Res* 79: 209–218.
- Larbi A, A Abadía, J Abadía, F Morales, 2006. Down co-regulation of light absorption, photochemistry, and carboxylation in Fe-deficient plants growing in different environments. *Photosynth Res*, 89:113–126.
- Lemerle D, R D Cousens, 1993. Responses of spring barley (*Hordeum vulgare* L.) and spring wheat (*Triticum aestivum* L.) to foliar or root entry of chlorsulfuron. *Weed Research*, 33: 335–344.
- McDonald AJS, T Ericsson, C-M Larsson, 1996. Plant nutrition, dry matter gain and partitioning at the whole-plant level. *J Exp Bot*, 47: 1245–1253.
- McKinney, 1941. Absorption of light by chlorophyll solutions. *J Biol Chem*, 140: 315–332.
- Morales F, N Moise, R Quílez, A Abadía, J Abadía, I Moya, 2001. Iron deficiency interrupts energy transfer from a disconnected part of the antenna to the rest of Photosystem II. *Photosynth Res*, 70: 207–220.
- Nenova V, 2006. Effect of iron supply on growth and photosystem II efficiency of pea plants. *Gen Appl Plant Physiol*, 32: 81–90.
- Nenova V, 2009. Growth and photosynthesis of pea plants under different iron supply. *Acta Physiol Plant*, 31: 385–391.

- Orcaray L, M Igal, A Zabalza, M Royuela, 2011. Role of exogenously supplied ferulic and p -coumaric acids in mimicking the mode of action of acetolactate synthase inhibiting herbicides. *J Agric Food Chem*, 59: 10162–10168.
- Osborne L D, A D Robson, 1993. What is the significance of herbicide-nutrient interactions on wheat? In: *Plant nutrition - from genetic engineering to field practice*, Ed. J. Barrow, Kluwer Academic Publishers, pp. 779–782.
- Osborne L D, A D Robson, D G Bowran, 1993. The impact of chlorsulfuron and diclof-methyl on nutrient-uptake by wheat. *Austr J Agric Res*, 44:1757–1766.
- Ozturk L, A Yazici, S Eker, O Gokmen, V Römheld, I Cakmak, 2008. Glyphosate inhibition of ferric reductase activity in iron deficient sunflower roots. *New Phytol*, 177: 899–906.
- Ray T, 1982. The mode of action of chlorsulfuron: A new herbicide for cereals. *Pesticide Biochem Phys*, 17: 10–17.
- Rengel Z, M S Wheal, 1997. Herbicide chlorsulfuron decreases growth of fine roots and micronutrient uptake in wheat genotypes. *J Exp Bot*, 48: 927–934.
- Roháček K, 2002. Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationships. *Photosynthetica* 40: 13–29.
- Sousa C P, J J O Pinto, E G Martinazzo, A T Perboni, M E Farias, M Bacarin, 2014. Chlorophyll a fluorescence in rice plants exposed of herbicides of group imidazolinon. *Planta daninha*, 32: 141–150.
- Stoyanova E, P Petrov, S Semerdjieva, 1997. Some effects of chlorsulfuron on the ultrastructure of root and leaf cells in pea plants. *J Plant Growth Reg*, 16: 1–5.
- Tang C, A D Robson, 2000. Cultivar variation in the effect of chlorsulfuron in depressing the uptake of copper in wheat. *Plant Soil*, 225: 11–20.
- Terry N, J Abadía, 1986. Function of iron in chloroplasts. *J Plant Nutr*, 9: 609–646.
- Wheal M, Z Rengel, 1997. Chlorsulfuron reduces rates of zinc uptake by wheat seedlings from solution culture. *Plant Soil*, 188: 309–317.
- Wheal M S, Z Rengel, R D Graham, 1999. Chlorsulfuron reduces extension of wheat root tips in low-zinc solution culture. *Annals of Botany*, 81: 385–389.
- Zabalza A, L Orcaray L, S Gaston, M Royuela, 2004. Carbohydrate accumulation in leaves of plants treated with the herbicide chlorsulfuron or imazethapyr is due to a decrease in sink strength. *J Agric Food Chem*, 52: 7601–7606.
- Zhou Q, W Liu, Y Zhang, K K Liu, 2007. Action mechanisms of acetolactate synthase-inhibiting herbicides. *Pestic Biochem Physiol*, 89: 89–96.